DOI: 10.1002/cbic.200700402

Transfer Printing of DNA by "Click" Chemistry

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This paper describes a straightforward procedure to immobilize oligonucleotides on glass substrates in well-defined micropatterns by microcontact printing with a dendrimer-modified stamp. The oligonucleotides are efficiently immobilized by "click" chemistry induced by microcontact printing. Acetylene-modified oligonucleotides were treated with an azide-terminated glass slide under the confinement of the dendrimer-modified stamp, without the use of a Cu^{I} catalyst. The immobilization is an irreversible, covalent, and one-step reaction that results in stable attachment of the oligonucleotides. Oligonucleotides with the acetylene-modification at the 5′ terminus hybridize selectively with full-length, complementary targets. Strands with more than one acetylene linker do not hybridize with complementary strands.

Microcontact printing (μCP) is a versatile tool for the patterning and functionalization of surfaces by direct synthesis within the confinement of the contact area of an elastomeric stamp.[1–3] Huck and co-workers have reported the synthesis of a RGD peptide and a 20-mer peptide nucleic acid (PNA) by microcontact printing.[4] Another example in which a poly(dimethylsiloxane) (PDMS) stamp was used as a tool to locally transfer and covalently bind enzymes was presented by Wilhelm and Wittstock.[5] Recently, we have demonstrated rapid imine formation through μCP of amines on aldehyde self-assembled monolayers and applied this chemistry to direct the immobilization of cytophilic proteins.[6,7] Moreover, we have shown that "click" chemistry can be applied in microcontact printing to couple alkynes to an azide-terminated substrate, *without* the need for a catalyst.[8]

"Click" chemistry was developed by Sharpless and co-workers as a modular approach to reliable chemical transformations that couple two molecules irreversibly under mild conditions.[9,10] The Huisgen 1,3-dipolar cycloaddition of alkynes and azides to yield triazoles is a typical example of a "click" reaction.[11–16] Azides and alkynes are easy to introduce also in biomolecules, and they are inert under physiological conditions.[17–22] In addition, the triazoles obtained by cycloaddition are also very stable, and almost impossible to oxidize or reduce.[13] This cycloaddition reaction is accelerated by a factor of 10^7 by Cu^{I} catalysis.[11,12]

Due to its biocompatibility, "click" chemistry is of interest for DNA-immobilization and DNA-modification strategies. "Click" chemistry was successfully applied by Chidsey and co-workers to surface immobilization of acetylene-bearing oligodeoxynucleotides onto azide-terminated surfaces in the presence of Cu^{I} tris(benzyltriazolylmethyl)amine (TBTA) catalyst.[23–25] Ju and co-workers have constructed a DNA microarray by using triazole formation to attach azido-modified DNA onto an alkyne-functionalized glass chip at room temperature under aqueous conditions in the presence of Cu^{I} as a catalyst.[26,27] The biocompatibility of the 1,3-dipolar cycloaddition and oligonucleotides was demonstrated in the postsynthetic labeling of alkyne-modified DNA.[28,29] Triazole formation has been applied to construct fluorescent oligonucleotides for DNA sequencing[17] and lately for the synthesis of multiply labeled carbohydrate oligonucleotides on solid supports.[30]

Recently, we have demonstrated that "click" chemistry can be applied in the direct μCP of acetylene-bearing molecules onto azide-terminated substrates *without* the need for a catalyst.[8] Here, we apply this observation to the μCP of oligonucleotides, without the use of a Cu^{I} catalyst. Using dendrimer-modified PDMS stamps, we printed oligonucleotides possessing acetylene units in their sequence onto azide-terminated glass slides to form patterns of oligonucleotides that are linked covalently to the substrate. For the development of biological arrays in particular, it is advantageous to exclude toxic Cu catalysts from the fabrication process.[31]

The azide-terminated substrate was prepared according to a literature procedure[32] by formation of an 11-bromoundecyltrichlorosilane monolayer on an activated glass slide and substitution of bromide with azide by treatment with a saturated solution of NaN_3. Modified oligodeoxynucleotides (ODNs) were synthesized with the acetylene unit in different positions of each strand (Table 1 and Scheme 1). In ODN-1, a single alkyne unit is positioned in the middle of the strand; in ODN-2, two

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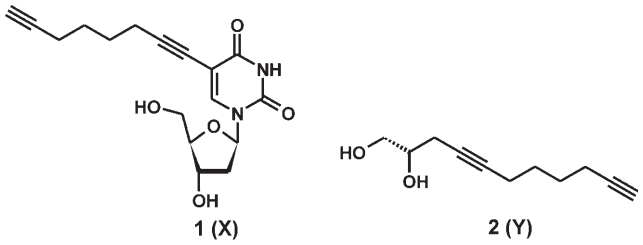
Table 1. Sequences of oligonucleotides containing acetylene-modified monomers **1** (**X**) and **2** (**Y**). Melting (T_m) and hybridization (T_H) temperatures of complementary strands.

Symbol	Sequence	T_m [°C]	T_H [°C]
ODN-1	5′-GCG CTG T**X**C ATT CGC G-3′		
ODN-2	5′-TTA ATT GAA TTC GAT T**X**G GGC CGG A**X**T TGT TTC-3′		
ODN-3	5′-**Y**GC GCT GTT CAT TCG CG-3′–fluorescein		
ODN-a	Cy5-5′-CGC GAA T-3′	15	2.0
ODN-b	Cy5-5′-CGC GAA TGA ACA CGC-3′	65.7	45.7
ODN-c	Cy5-5′-GAA TTC AAT TAA-3′	26.2	6.2
ODN-d	Cy5-5′-GAA ACA ATC CGG CCC AAT CGA ATT CAA TTA A-3′	75.3	56.3
ODN-e	Cy5-5′-CGC GAA TGA ACA GCG C-3′	65.7	45.7



Scheme 1. Monomers used in the solid-phase synthesis of acetylene-modified oligonucleotides.

acetylene units are at two locations in the middle of the strand; and in ODN-3, a single unit is located at the 5′ terminus. ODN-1 is complementary to ODN-a (fragment) and ODN-b (full length). ODN-2 is complementary to ODN-c (fragment) and ODN-d (full length). ODN-3 is complementary to ODN-e (full length).

We have developed an efficient method for transferring DNA to a substrate and facilitating the positioning of DNA with high lateral resolution by μCP based on the modification of PDMS stamps with dendrimers ("dendri-stamps", Figure 1).[33] This protocol is based on the electrostatic interaction between a negatively charged, oxidized PDMS stamp and positively charged dendrimers (polypropyleneimine tetrahexacontaamine dendrimer, G5-PPI). G5-PPI possesses 64 amino groups at the periphery, thus providing a high density of terminal ammonium groups at neutral pH. All types of nucleic acid, including plasmid DNA, single- and double-stranded oligonucleotides, and RNA, can form complexes through electrostatic interactions with PPI or PAMAM dendrimers.[34] The binding of DNA molecules to the stamp surface is a prerequisite for their transfer to the substrate. Modification of the PDMS stamp with G5-PPI dendrimers gives a high density of positive charge on the stamp surface that can attract negatively charged DNA molecules in a "layer-by-layer" arrangement.[35] After incubation of acetylene-modified oligonucleotides (ODN-1, ODN-2 and ODN-3) with the dendri-stamp and drying with nitrogen, the inked stamp (*without* Cu^I catalyst) was brought into conformal contact with the azide-terminated substrate for 1 h at room temperature with a weight of 120 g to ensure the contact between the stamp and the substrate. Subsequently, the stamp was removed, and the substrate was rinsed thoroughly with ethanol containing triethylamine to remove any residue of dendrimers.[36]

In order to visualize the printed pattern of oligonucleotides, strands that did not possess fluorescent dyes in their structure (ODN-1 and ODN-2) were subjected, after printing, to binding by fluorescent molecules (TOTO-1). This dye is known into bind to single-stranded DNA. After binding, the substrates were imaged by using a laser-scanning fluorescent confocal microscope. The topological pattern of the stamp was faithfully replicated as a line or dot pattern of immobilized, fluorescent oligonucleotides (Figure 2). The signal-to-noise ratio, as measured by a line scan, was invariably higher than 100, and often higher than 1000. These patterns were also investigated with an atomic force microscope. The pattern comprised homogeneously distributed oligonucleotides forming a uniform coating with an average height of printed patterns of 3 nm (Figure 3). The height of the pattern is consistent with a monolayer of oligonucleotides.

To verify that the cycloaddition reaction between alkyne and azide is essential for efficient transfer printing of the oligonucleotides on the substrate, we printed a fluorescent oligonucleotide similar to ODN-3 but without alkyne-modified mono-

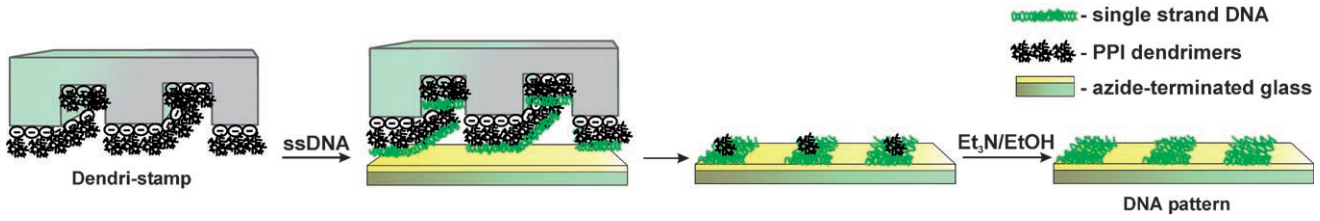


Figure 1. Schematic representation of transfer printing of DNA with a dendri-stamp. An oxidized PDMS stamp is first inked with dendrimers and subsequently incubated with DNA. A "click" reaction between acetylene-modified DNA and azide-terminated substrate is induced in the contact area between stamp and substrate. After printing, the substrate is rinsed with EtOH/Et_3N to wash residual dendrimers from the DNA substrate.

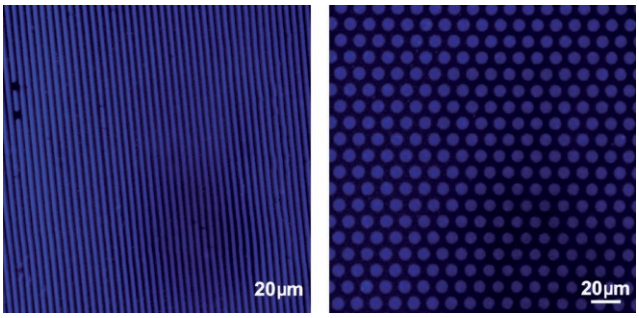


Figure 2. Fluorescent image of ODN-1 (left) and ODN-2 (right) printed with dendri-stamps on azide-terminated glass slides, after binding of TOTO-1 dye.

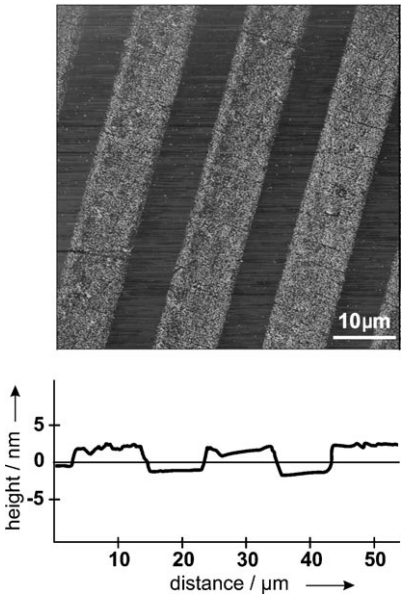


Figure 3. AFM tapping-mode image of ODN-3 printed with dendri-stamps on azide-terminated glass slides.

mer. When this oligonucleotide was printed on an azide substrate, no significant immobilization was observed (see the Supporting Information). We also observed that the contact time is a critical parameter in the transfer printing process. After 1 hour of contact time, the oligonucleotides are irreversibly attached to the substrate and not susceptible to sonication or rinsing. The oligonucleotide density was lower at shorter contact times. This observation is diagnostic of a chemical reaction with a particular kinetics, rather than diffusion-controlled physisorption.

Subsequently, the immobilized oligonucleotides were hybridized with complementary strands. To investigate whether immobilization through triazole linkage in the middle of the strand affects the subsequent hybridization, we investigated this reassociation with complementary strands of different length (Figure 4). In the cases of ODN-1 and ODN-2, the hybridization was examined with two different strands: with fragment strands (ODN-a, ODN-c) and with full-length strands that had the same number of oligonucleotides as the probes (ODN-b, ODN-d), respectively. ODN-1 and ODN-2 did not hybridize with full-length, complementary strands (ODN-b, ODN-d); however, ODN-1 hybridized with the short fragment of complementary, Cy5-labeled strand ODN-a. The strands that hybridized successfully showed a visible, fluorescent pattern (Figure 5).

The hybridization of ODN-2 with short, complementary ODN-c was not successful. The short fragment of complementary strand probably has too low a GC content to be hybridized. Attempts to hybridize the full-length complementary strand resulted in patterns that were incomplete and had a variable level of intensity; this could be due to the double point of immobilization of the probe strand or due to the highly unfavorable formation of the double-strand complex parallel to the surface. Finally, immobilized probe ODN-3 hybridizes readily with the full-length, complementary, Cy5-labeled ODN-e strand (Figure 6); this could be expected since the immobilization point of ODN-3 is located at the terminus of the strand. The signal-to-noise ratio of the hybridized oligonucleotide pattern as measured by a line scan was invariably higher than 100, and often higher than 1000. These hybridiza-

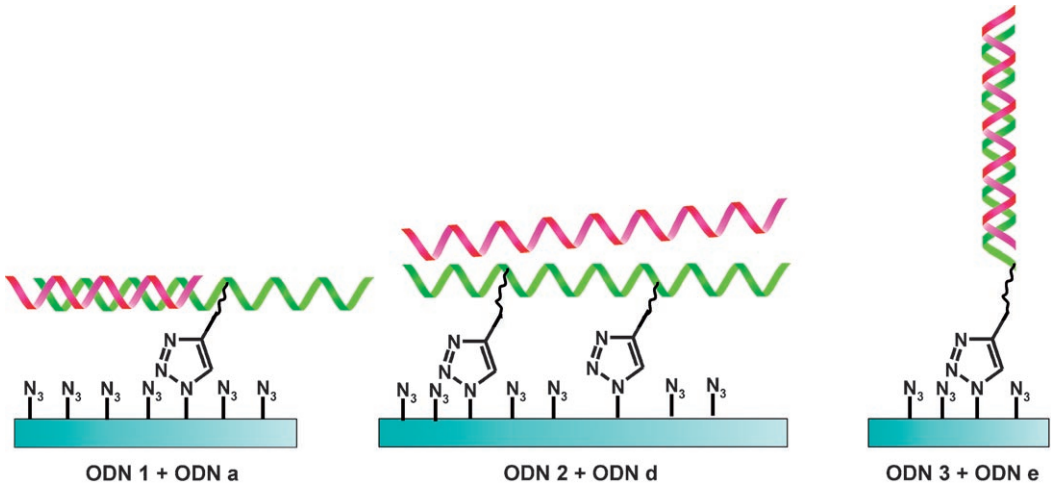


Figure 4. Immobilization of oligonucleotides ODN-1, ODN-2, and ODN-3, which possess acetylene units in different positions, and hybridization with complementary strands ODN-a, ODN-d, and ODN-e.

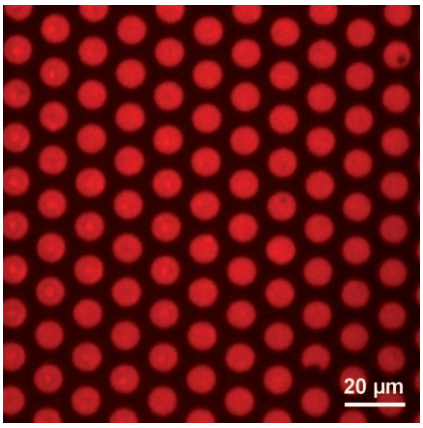


Figure 5. Fluorescent image of ODN-1 printed with a dendri-stamp and hybridized with a complementary, Cy5-labeled ODN-a strand.

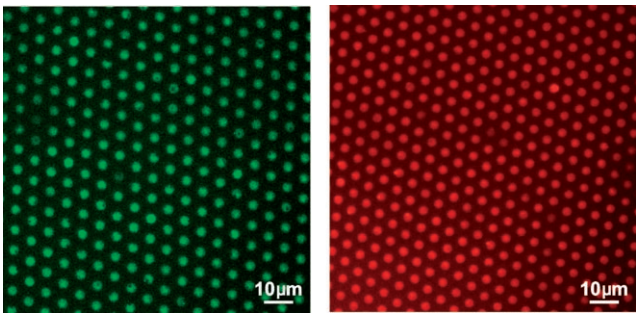


Figure 6. ODN-3 patterns made by microcontact printing with dendri-stamps. Fluorescein-labeled probe before (left) and after hybridization with the complementary Cy5-labeled probe ODN-e (right).

tion experiments also confirm that no significant amount of dendrimer is left on the substrate, since residual dendrimers would likely bind any nucleotide sequence in an unspecific (electrostatic) manner.

In summary, we have developed a simple and straightforward procedure to immobilize oligonucleotides on glass slides in well-defined patterns by μCP using a dendri-stamp through "click" chemistry without the use of a Cu^{I} catalyst. Acetylene-modified oligonucleotides were treated with an azide-terminated glass slide within the confinement of a dendrimer-modified stamp. The immobilization is an irreversible, covalent, and one-step reaction that results in stable attachment of oligonucleotides. The oligonucleotide strand with the acetylene-modification at the 5′ terminus gave the best result in hybridization with full-length, complementary targets. Strands with more than one acetylene linker showed a lack of hybridization.

In comparison to other types of immobilization reactions, the advantage of the click-chemistry approach is that it is a one-step, covalent immobilization that proceeds in high yield, irrespective of the medium, without producing any by-products, and without the need for a catalyst. Another main advantage is that the required azide and alkyne reactive groups as well as the resulting triazole link are biocompatible, stable, and orthogonal to any typical biochemical bonds and interactions, so this chemistry does not interfere or crossover with any bioactivity. This orthogonality implies that the link can be introduced at a very specific location in the oligonucleotide, and the molecule can be immobilized in a very specific orientation. We contend that this method can be extended to other (bio)molecules that are modified with an acetylene unit and can be attached to azide-terminated monolayers. Moreover, this strategy can be utilized in microarray fabrication in combination with microfluidic devices or robotic spotters, and in studies of the influence of the linker and its position on probe immobilization and hybridization, which are critical factors in microarrays and biosensors.

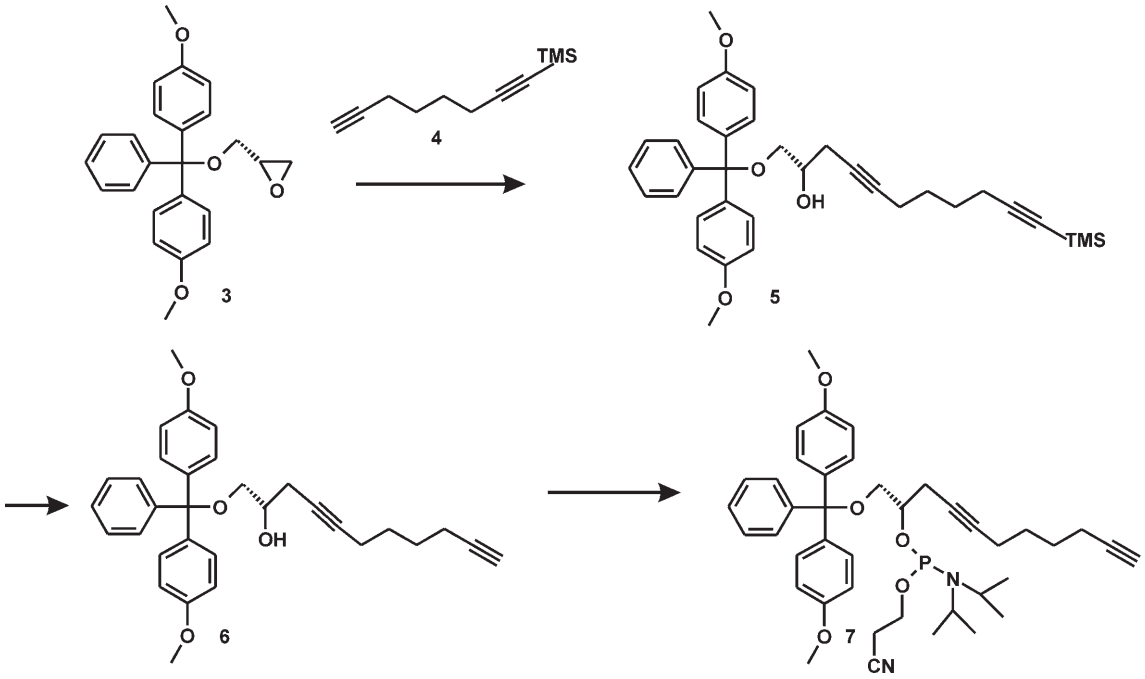
Experimental Section

Materials: Probes ODN-1, ODN-2, and ODN-3 employed in surface studies were synthesized according to the procedures listed below. The complementary strands were purchased from Sigma. The target sequences were Cy5–5′-ACA GCG C-3′, Cy5–5′-CGC GAA TGA-ACA GCG C-3′, Cy5–5′-GAA ACA ATC CGG CCC AAT CGA ATT CAA TTA A-3′, Cy5–5′-AAT CGA ATT CAA TTA A-3′, Cy5–5′-CGC GAA TGA ACA GCG C-3′. All the nucleotides were HPLC purified and modified by the manufacturer. The probe concentration was 1 μM in Tris-EDTA buffer (pH 8), and the target concentration was 1 μM in 4×SSC, 0.2% SDS solution. Before use, oligonucleotides were denatured at 95 °C for 5 min. All buffers and immobilization solutions were prepared with 18 MΩ cm distilled water (MilliQ). The following materials and chemicals were used as received: PDMS (Dow Corning), polypropyleneimine tetrahexacontaamine dendrimers, generation 5 (Aldrich), NaN_3, 11-bromoundecyltrichlorosilane (Sigma). All solvents were HPLC grade, and all other reagents were analytical grade. Other solvents or reagents were purchased from either Aldrich or Sigma.

Synthesis of monomers and oligonucleotides: The oligonucleotides were synthesized "DMT-on" according to literature procedures.[28] The solid-phase building block for monomer **1** was also synthesized according to literature procedures.[28] The building block for monomer **2** was synthesized according to Scheme 2.

Compound ***5***:[37] *n*-Butyl lithium (360 μL, 0.9 mmol, 1.2 equiv) was added to a cooled (−78 °C) solution of 1-trimethylsilyl-1,7-octadiyne (**4**)[28] (159 mg, 0.9 mmol, 1.2 equiv) in diethyl ether within 5 min. The reaction mixture was stirred for an additional 20 min. Then $BF_3{\cdot}Et_2O$ (90 μL, 0.9 mmol, 1.2 equiv) was added within 2 min, and the mixture was stirred for an additional 20 min. Then a solution of (*R*)-1-(dimethoxytritylmethoxymethyl)oxirane (**3**)[38] (281 mg, 0.75 mmol, 1.0 equiv) in diethyl ether was added, and the mixture was stirred for 2 h at −78 °C. The reaction was quenched with saturated aq. $NaHCO_3$, and the solution was allowed to warm to room temperature. The product was extracted with diethyl ether (3×), and the organic phases were washed with water (3×) and brine (3×). The solvents were removed in vacuo, and the crude product was purified by column chromatography (hexane with 10→15% ethyl acetate). Product **5** was obtained as colorless oil (414 mg, 99%) and used directly in the next step. R_f = 0.22 (hexane/20% EtOAc).

Compound ***6***: Compound **5** (414 mg, 0.75 mmol) was dissolved in THF/MeOH (1:1; 20 mL), dry K_2CO_3 (515 mg, 3.74 mmol, 5.0 equiv) was added, and the reaction mixture was stirred overnight. The mixture was diluted with MeOH (200 mL), filtered through celite, and concentrated. Column chromatography (hexane with 20% ethyl acetate + 1% pyridine) afforded product **6** (110 mg, 29%).



Scheme 2. Synthesis of the solid-phase building block for monomer **2**.

$R_f = 0.2$ (hexane with 20% ethyl acetate); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 1.57–1.50$ (m, 4H; CH_2–CH_2), 1.94 (t, $J = 2.63$, 2.63 Hz, 1H; C≡CH), 2.14 (td, $J = 8.50$, 3.25 Hz, 2H; CH_2), 2.17 (dt, $J = 6.56$, 2.57 Hz, 2H; CH_2), 2.34 (d, $J = 4.96$ Hz, 1H; OH), 2.41–2.45 (m, 2H; CH_2), 3.21 (ddd, $J = 15.37$, 9.32, 5.31 Hz, 2H; CH_2), 3.79 (s, 6H; CH_3), 3.84–3.90 (m, 1H; CH), 6.85–6.80 (m, 4H), 7.21 (t, $J = 7.32$ Hz, 1H), 7.27–7.33 (m, 6H), 7.45–7.41 (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$): $\delta = 17.9$ (CH_2), 18.2 (CH_2), 24.2 (CH_2), 27.5 (CH_2), 27.8 (CH_2), 55.2 (CH_3), 66.0 (CH_2), 68.4, 69.6 (CH), 76.0, 82.2, 84.2, 86.1, 113.1 (CH), 126.8 (CH), 127.8 (CH), 128.1, (CH), 130.0 (CH), 136.0, 144.8, 158.5; HR-MS (ESI-FT-ICR$^+$): calcd for $C_{32}H_{34}O_4Na$: 505.2349 $[M+Na]^+$; found 505.2329.

Compound ***7***: *N,N′*-Diisopropylethylamine (72 μL, 0.42 mmol, 2.0 equiv) was added to a solution of **6** (100 mg, 0.21 mmol, 1.0 equiv) in dichloromethane (3 mL), and the reaction mixture was stirred for 10 min at room temperature. Then 2-cyanoethoxy-*N,N′*-diisopropylchlorophosphoramidite (70 μL, 0.31 mmol, 1.5 equiv) was added, and the mixture was stirred for 1.5 h. The solvent was removed under vacuum, and the crude product was purified by column chromatography (deactivated silica; hexane with 10% ethyl acetate + 1% pyridine). Product **7** was obtained as a mixture of diastereomers (123 mg, 86%). ^{31}P NMR (81 MHz, CD_2Cl_2): $\delta =$ 149.59, 149.67.

Modification of glass slides: Clean microscope cover glass (Paul Marienfeld Gmbh & Co.KG, Germany) was activated with piranha solution (concentrated H_2SO_4 and 33% aqueous H_2O_2 in a 3:1 ratio) for 45 min. ***CAUTION!*** Piranha solution should be handled with care as it has been reported to detonate unexpectedly. The glass was rinsed with water (MilliQ) and immediately immersed in 11-bromoundecyltrichlorosilane in toluene (0.1 vol%) for 20 min at 20 °C. Following monolayer formation, the substrates were rinsed with toluene to remove any excess of silanes and subsequently dried under N_2. Bromide was substituted for the azide in a saturated solution of NaN_3 in DMF for 48 h at 70 °C.[32] The substrate was rinsed with MilliQ water and ethanol and dried under nitrogen.

Fabrication of stamps: Silicon wafer-based masters with etched structures were prepared by UV photolithography. The master surface was fluorinated with fluorosilanes. PDMS stamps were fabricated by curing Sylgard 184 on the surface of the master at 60 °C for 12 h.

Microcontact printing of DNA with dendrimers: PDMS stamps were first oxidized in a UV/plasma reactor (Ultra-Violet Products, model PR-100) for 30 min at a distance of about 2 cm from the plasma source. This reactor contains a low-pressure mercury lamp that emits UV light at 185 nm (1.5 mW cm^{-2}) and 254 nm (15 mW cm^{-2}). Subsequently, the hydrophilic stamps were immersed in an ethanolic solution of G5-PPI dendrimers (1 μM) for 30 s and blow-dried with nitrogen. A drop of oligonucleotide solution was incubated on the stamp for 20 min at room temperature. The probe concentration was 1 μM in Tris-EDTA buffer pH 8 solution. The stamp was dried with nitrogen and brought into conformal contact with the azide-terminated glass slide for 1 h under a load of 120 g. After printing, the stamp was lifted off, and the substrate was rinsed with ethanol (30 mL) containing a drop of triethylamine in order to remove the dendrimer layer and subsequently dried with nitrogen.

Binding of TOTO-1 to the patterned oligonucleotides: A solution of TOTO-1 in DMSO (1 μM) was dropped onto the glass slide patterned with oligonucleotide for 5 min; then the slides were rinsed with DMSO, ethanol, and water. The substrate was dried with nitrogen.

Hybridization on substrate surface: For hybridization, a 5′-Cy5-labeled oligonucleotide was diluted to 1 μM in 4×SSC containing 0.2% SDS, and applied to the surface of the modified glass slide, as described by Afanassiev et al.[39] A coverslip was placed gently on top of the solution, and the substrates were transferred to the hybridization oven overnight (the hybridization temperatures are listed in Table 1). The unhybridized probes were removed by washing with vigorous agitation in 1×SSC with 0.1% SDS solution for

5 min at the hybridization temperature, 0.1×SSC with 0.1% SDS for 5 min at RT, and subsequent washing in water for 5 min. After washing, the glass slides were dried under nitrogen and scanned on a confocal fluorescent microscope (Zeiss 510) to visualize hybridization signals.

Atomic force microscopy: AFM measurements were carried out on a Dimension 3100/Nanoscope IVa (Digital Instruments, Santa Barbara, CA, USA) in tapping mode, with 512×512 data acquisitions, by using ultrasharp tips (MikroMash, Spain). All imaging was conducted at room temperature in air.

Fluorescence microscopy: Fluorescent images were acquired with a Carl Zeiss LSM 510 scanning confocal microscope. Red- and green-labeled DNA molecules were visualized at $\lambda_{ex}=650$ nm ($\lambda_{em}=670$–700 nm) and $\lambda_{ex}=495$ nm ($\lambda_{em}=517$ nm), respectively. The emitted fluorescence was collected on a R6357 spectrophotometer.

Acknowledgements

This work was supported by NanoImpuls/NanoNed, the nanotechnology program of the Dutch Ministry of Economic Affairs (grant TTF6329).

Keywords: cycloadditions · dendrimers · DNA · microcontact printing · surface chemistry

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Received: July 20, 2007
Published online on September 13, 2007

